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HPV16 E7 oncoprotein deregulates B-*myb* expression: correlation with targeting of p107/E2F complexes

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HPV16 is a human tumour virus encoding two principal oncoproteins, E6 and E7. Expression of E7 can induce DNA synthesis in quiescent cells and this property coincides with its ability to bind to the cell proteins pRb and p107. As these cell proteins are regulators of the transcription factor E2F, we have investigated whether the interaction with E7 could result in induction of cell cycle regulated genes. We show that B-*myb*, whose induction at the G_1/S boundary is regulated by release from E2F mediated transcriptional repression, is a target for transcriptional activation by E7 and is the first E7 responsive cell gene to be identified. E7 transactivation leads to both inappropriate transcription of B-*myb* during G_1 and constitutive over-expression in cycling cells. B-Myb plays an essential role in cell cycle progression, and activation by E7 is likely to contribute to the mitogenic activity of the viral oncoprotein. Regulation of the B-*myb* promoter in NIH3T3 cells correlates with binding of distinct p107-containing complexes at the E2F binding site, and analysis of E7 mutants confirms that B-*myb* transcription in these cells is regulated through interactions with p107 rather than pRb. These results provide the first example of a potentially specific role for p107 in the regulation of the cell cycle.

Key words: B-*myb*/E2F/HPV16 E7/p107/pRb

Introduction

The papillomaviruses are epitheliotropic viruses which normally cause benign proliferations or warts (von Knebel Doeberitz, 1992). Infection with certain types of these viruses, however, gives rise to lesions with a potential for malignant progression, and infection by the so-called high risk genital human papillomavirus (HPV) types is thought to play a role in the development of almost all cervical cancers (zur Hausen, 1991). There is considerable evidence that the oncogenic potential of these viruses is related to the expression of two small viral proteins, E6 and E7, both of which show transforming and immortalizing activities in rodent and human cells in culture (Vousden, 1991). There is also evidence that the E7 protein plays an important role in the normal viral life cycle (Brandsma *et al.*, 1991), and it is possible that the ability of E7 to induce proliferation or prevent the normal programme of epithelial cell differentiation contributes to both viral replication and, under some circumstances, malignant progression.

The E7 protein encoded by HPV16, the most commonly detected high risk genital HPV type, associates with the cell-encoded retinoblastoma protein (pRb) and with the pRb-related p107 protein (Dyson *et al.*, 1989; Davies *et al.*, 1993). Although these activities are not essential for all E7 functions they appear to be important, but not sufficient, for E7 to transform rodent cells in culture (Edmonds and Vousden, 1989; Watanabe *et al.*, 1990; Phelps *et al.*, 1992). The contribution of pRb and p107 interactions to E7 transformation is not clear, but may be related to the ability of E7 to induce DNA synthesis in quiescent rodent fibroblasts (Banks *et al.*, 1990b; Watanabe *et al.*, 1992). The potential consequences of complex formation between E7 and pRb/p107 became apparent following the identification of these cell proteins as regulators of transcription factors such as E2F (Hiebert *et al.*, 1992; Weintraub *et al.*, 1992; Zamanian and La Thangue, 1992; Ogris *et al.*, 1993; Schwarz *et al.*, 1993; Zhu *et al.*, 1993). The transcription activation activity of E2F is inhibited in complexes with pRb or p107, and these interactions may therefore account for the tight cell cycle regulated transcription of several genes shown to contain E2F binding sites within their regulatory elements (Mudryj *et al.*, 1990; Dalton, 1992; Ogris *et al.*, 1993; Slansky *et al.*, 1993). The observation that E7 can disrupt complexes between E2F and pRb or related proteins in mouse and human cells (Phelps *et al.*, 1991; Chellappan *et al.*, 1992; Pagano *et al.*, 1992; Morris *et al.*, 1993), suggested that the consequence of these viral-host protein interactions could be deregulated expression of E2F-responsive genes. Consistent with this model, expression of E7 resulted in transactivation of the E2F-regulated adenovirus E2 promoter (Phelps *et al.*, 1988); mutational analyses showed that this activity was dependent on the ability of E7 to bind pRb (Edmonds and Vousden, 1989; Watanabe *et al.*, 1990; Phelps *et al.*, 1992), therefore suggesting that it resulted from disruption of inhibitory complexes between E2F and pRb or related cell proteins. To this point, however, no cell genes have been identified whose transcription is regulated by E7 expression.

B-Myb is a relative of the transcription factor c-Myb and appears to be expressed in all cell lineages (Nomura *et al.*, 1988). In both haematopoietic and fibroblast cells, transcription of the B-*myb* gene was found to be strictly regulated during the cell cycle, being induced to a high level only late in G_1 and during S phase (Golay *et al.*, 1991; Reiss *et al.*, 1991; Lam *et al.*, 1992; Lam and Watson, 1993). We have shown previously that the negligible transcription of B-*myb* observed in NIH3T3 fibroblasts either during quiescence or in the early stages of G_1 was the result of negative regulation mediated through an E2F binding site within the B-*myb* promoter (Lam and Watson, 1993). Mutation of this site in luciferase reporter plasmids resulted

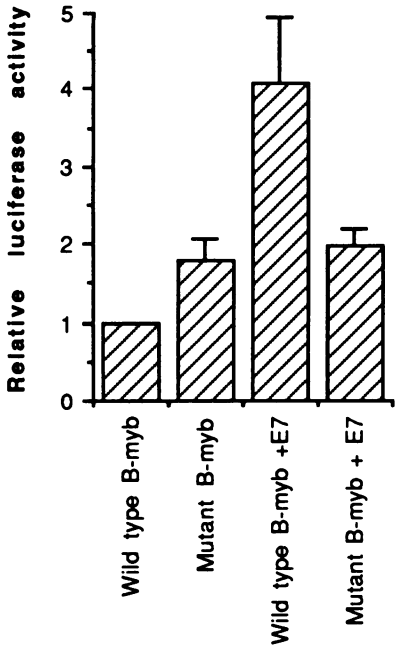

Fig. 1. Transactivation by HPV16 E7 of the wild-type mouse B-*myb* promoter or a mutant promoter lacking the E2F binding site. Cycling NIH3T3 cells were transfected with luciferase reporters driven by wild-type or mutant B-*myb* promoters, pGL2-(-536) and pGL2-(-536)mut respectively, either in the presence of the E7 expression plasmid pMo16E7 or the empty vector pMo. Luciferase activity was measured in cell extracts made 48 h after transfection. The results represent the mean of at least six experiments.

in high levels of B-*myb* promoter activity throughout the cell cycle. Analysis of NIH3T3 proteins binding to the B-*myb* promoter E2F site revealed a specific G_0/G_1 complex which was replaced by a distinct complex late in G_1 and during S phase (Lam and Watson, 1993). Disappearance of the G_0/G_1 complex correlated with induction of B-*myb* transcription, suggesting that repression of promoter activity at this stage of the cell cycle is imposed by binding of this complex to the B-*myb* E2F site. Since B-*myb* expression is necessary for cell growth (Arsura *et al.*, 1992; Sala and Calabretta, 1992), it is possible that B-Myb represents an important regulator of proliferation with a role in controlling entry into DNA synthesis.

In this study we demonstrate transcriptional transactivation of B-*myb* by HPV16 E7 and provide evidence that in NIH3T3 cells this is a direct result of interference with an E2F complex containing p107. The potential importance of B-Myb in the regulation of cell cycle progression suggests that this activity might contribute to the ability of E7 to induce DNA synthesis and consequent deregulation of cell growth.

Results

E7 positively regulates the mouse B-myb promoter

Previous studies have shown that transcription from the mouse B-*myb* promoter is cell cycle regulated and that the repression of transcription seen during the early stages of the cell cycle is dependent on an E2F binding site (Lam and Watson, 1993). Repression of B-*myb* transcription in G_0/early G_1 was seen to correlate with the presence of a distinct E2F complex in NIH3T3 cells which, by reference to other studies, could involve p107 (Schwarz *et al.*, 1993) or possibly other pRb-related proteins (Chittenden *et al.*, 1993). As E7 is potentially able to displace E2F from complexes with these proteins, co-transfection experiments were performed to determine whether expression of E7 in cells could lead to activation of the B-*myb* promoter. Co-expression of E7 and a B-*myb* luciferase reporter in asynchronous cycling NIH3T3 cells was indeed found to result in a 4-fold activation of transcription from the wild-type B-*myb* promoter (Figure 1, lanes 1 and 3). In contrast, the activity of the mutant promoter was not affected by E7 (Figure 1, lanes 2 and 4). As described previously (Lam and Watson, 1993), mutation of the B-*myb* E2F site resulted in higher promoter activity in cycling cells (Figure 1), presumably because an inhibitory E2F complex present in G_1 was unable to bind. The effect of E7 on the wild-type promoter was not dependent solely on derepression of this inhibition, however, as the presence of the E2F binding site permitted transactivation of the wild-type promoter to a level ~2-fold greater than that obtained with the mutant promoter (Figure 1, lanes 2 and 3).

Activation of endogenous B-myb expression depends on pRb/p107 binding activity of E7

Since E7 could be shown to transactivate transcription from the B-*myb* promoter in a transient assay, we next examined whether E7 could also induce expression of endogenous B-*myb*. For this analysis, we took advantage of previously characterized NIH3T3 cell lines which constitutively express wild-type or mutant E7 from the Moloney murine leukaemia virus LTR (Edmonds and Vousden, 1989; Davies *et al.*, 1993). This analysis (Figure 2A) showed increased levels of B-*myb* mRNA in two cell lines expressing wild-type E7 (E7/2 and E7/5) when compared with control cells (pMo, transfected with the empty vector only). The E7/2 cells expressed E7 protein at far greater levels than did E7/5 cells (Figure 2B), and the extent of B-*myb* mRNA over-expression (4.5-fold and 1.6-fold, respectively) was seen to correlate with E7 protein levels (Figure 2B). This effect did not result from differences in cell cycle progression as the various lines have similar doubling times (data not shown). As transactivation of the B-*myb* promoter by E7 was found to be dependent on the E2F binding site (Figure 1), it is likely that this E7 activity is related to its interaction with pRb and p107. To provide evidence for this interaction, B-*myb* mRNAs were also analysed in two cell lines expressing mutant E7 proteins which differ in their ability to bind pRb/p107; the mutant proteins were expressed at levels comparable with E7/5 cells (Figure 2B). It was found that cells expressing an E7 mutant (PRO2) which retains the ability to bind pRb/p107 also showed over-expression of B-*myb* (by ~2.2-fold), whereas no increase in B-*myb* expression was seen in the cells containing an E7 mutant (GLY24) unable to bind pRb/p107 (Figure 2A). These results suggest a correlation between the ability of E7 to bind pRb/p107 and deregulate B-*myb* expression. These functions of E7 do not strictly correlate with transforming activity since the PRO2 mutant, which behaved like wild-type E7 in inducing over-expression of B-*myb*, was unable to transform NIH3T3 cells to anchorage independent growth (Figure 2C). This effect on B-*myb* expression does, however, correlate with the ability of E7 to induce DNA synthesis in NIH3T3 cells (Banks *et al.*, 1990b).

E7 activation of B-myb expression during early G_1

Although these studies indicated that E7 can alter B-*myb* expression, it was important to determine whether this was

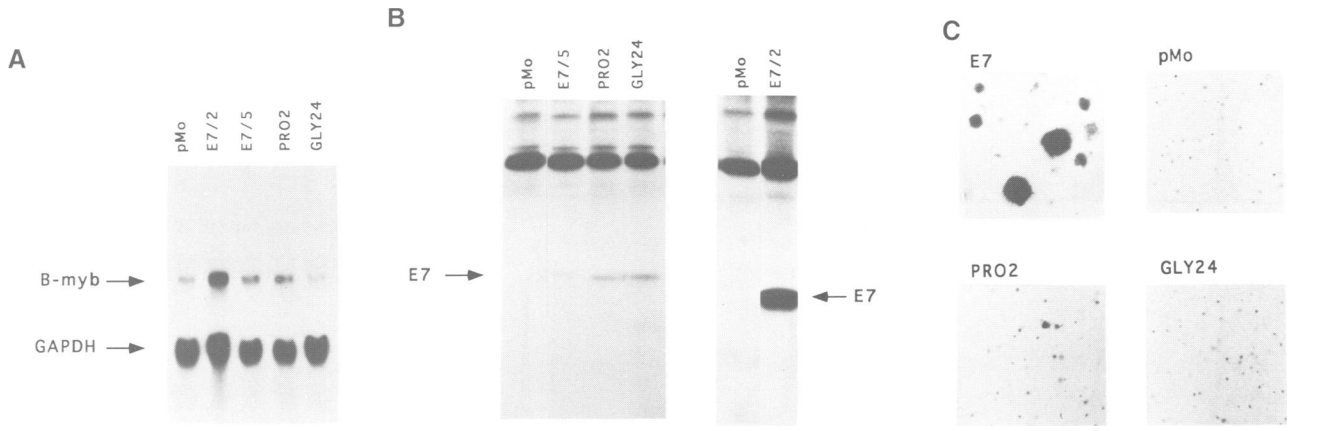

Fig. 2. (**A**) B-*myb* expression in E7-transfected NIH3T3 cells. Cytoplasmic RNA was extracted from cycling cells stably transfected with pMo vector sequences only (pMo) or from cells expressing wild-type E7 (E7/2 and E7/5) and E7 mutants (PRO2 and GLY24). RNAs were detected by Northern blot analysis, probing for B-*myb* and GAPDH expression simultaneously. (**B**) Comparison of E7 protein expression in the stably transfected NIH3T3 cell lines. Immunoprecipitation of E7 protein from metabolically labelled cell extracts showed comparable levels of E7 expression in E7/5, PRO2 and GLY24 cells, with considerably higher levels of expression in E7/2. (**C**) Transformation assay of the NIH3T3 cell lines expressing wild-type or mutant E7 proteins by culture in semi-solid medium. Only cells expressing wild-type E7 showed anchorage independent growth.

a direct effect or an indirect consequence of the ability of E7 to perturb the normal regulation of cell cycle progression. In order to address this point, we examined the NIH3T3 line expressing high levels of E7 (E7/2) more closely. Although these cells fail to arrest synchronously in G_0 upon serum-deprivation (Davies *et al.*, 1993), cell cycle analysis following nocodazole treatment revealed that these cells were synchronously released from the mitotic block and showed no marked difference from controls in their rate of progress through G_1 and into DNA synthesis (Figure 3A). Mitotic arrest was used, therefore, to synchronize E7/2 and control pMo cells to allow a comparison of B-*myb* mRNA abundance at the G_1 stage of the cell cycle. Previous studies had shown that B-*myb* is expressed at high levels in nocodazole-arrested cells (Lam *et al.*, 1992). Upon release into G_1, transcript levels were found to diminish substantially until reaching a low at ~6 h, presumably reflecting decay of pre-existing B-*myb* mRNA in early G_1; subsequently, transcript abundance increased as cells reached late G_1 and then entered S phase (Lam *et al.*, 1992). Consistent with published data, B-*myb* mRNA abundance was very low in control pMo cells at 6 h after removal of nocodazole and then showed a substantial increase, beginning at 8 h after nocodazole removal, as the cells progressed through the cycle (Figure 3B). In contrast to control cells, the E7/2 cells expressed high levels of B-*myb* transcripts regardless of their position in the cell cycle (Figure 3B). Thus, B-*myb* mRNA was abundant in E7/2 cells at 6 h after removal of the drug when the cells were in mid G_1 (Figure 3B). It is notable, moreover, that even during S phase (i.e. at the 10 h and 12 h points) B-*myb* mRNA levels were appreciably greater in the E7/2 cells than in the pMo controls (Figure 3B). These data indicate that expression of E7 leads to both inappropriate transcription of B-*myb* in the G_1 phase of the cell cycle and also to overexpression of this cellular gene in S phase.

E2F complexes in NIH3T3 cells contain p107 and cyclin A

As transactivation of B-*myb* transcription by E7 was dependent on the B-*myb* promoter E2F site (Figure 1), it appeared likely that induction of B-*myb* transcription in E7/2 cells resulted from the interaction of E7 with higher order E2F complexes containing pRb or related proteins. One possible consequence of this interaction could be disruption of the G_0/G_1 E2F complex which has been previously associated with repression of B-*myb* promoter activity in NIH3T3 cells (Lam and Watson, 1993). As a first step towards addressing this possibility, we further analysed the components of the distinct G_0/G_1 and S phase E2F complexes in untransfected NIH3T3 cells. These studies utilized a series of antibodies to compare the complexes present in quiescent NIH3T3 cells with those found at 16 h following release of these cells from serum-starvation (Lam and Watson, 1993). Both the G_0/G_1 and S phase complexes were super-shifted by a p107-specific monoclonal antibody (SD15) (Figure 4), demonstrating the presence of this protein in both these complexes. Addition of a cyclin A antibody had no effect on the G_0/G_1 complex but significantly inhibited DNA binding of the S phase complex (Figure 4). Neither complex was affected by a pRb antibody. In all, several pRb antibodies were tested, including XZ104 (Hu *et al.*, 1991) which efficiently recognizes mouse pRb in immunoprecipitations and which super-shifts a pRb/E2F complex in human cell lines (data not shown). These studies therefore point to the absence of a detectable pRb/E2F complex in NIH3T3 cells.

E2F complexes are targeted in E7/2 cells

We next examined whether the G_0/G_1 and S phase p107/E2F complexes were affected by expression of E7 in NIH3T3 cells. To analyse complexes in G_1 cells, whole cell extracts were made at 6 h after release from nocodazole arrest from control pMo cells and from E7/2 cells. Three specific protein−DNA complexes were detected in the 6 h pMo cell extract. These comprised the free E2F complex and two higher order complexes which corresponded in mobility to the G_0/G_1 and S phase complexes (Figure 5A). As expected, the higher order complexes were both super-shifted by addition of the p107 antibody (Figure 5A). The apparent presence of the S phase complex in these cell extracts may reflect conversion of the preponderant G_1 complex by excess cyclin A present in a few contaminating S phase cells or, alternatively, may reflect a distinct G_1 complex containing cyclin E (Lees *et al.*, 1992). This pattern

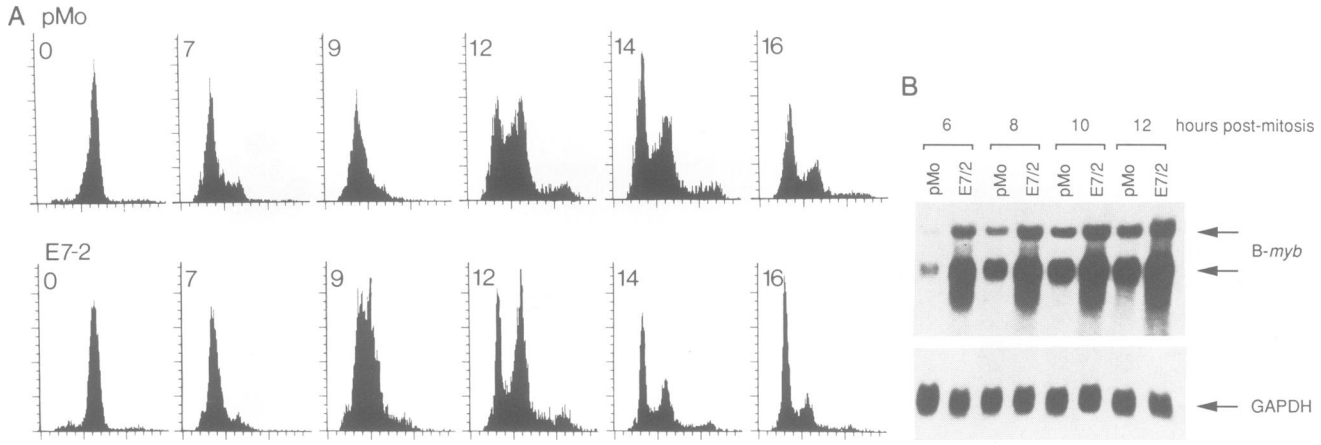

Fig. 3. Expression of B-*myb* in control and E7-transfected NIH3T3 cells synchronized by nocodazole arrest in G_2/M. (**A**) Cell cycle status of control (pMo) and and E7-transfected (E7/2) NIH3T3 cells. Relative DNA content was analysed by flow cytometry at the indicated time in hours after removal of nocodazole. Both cell lines start to enter DNA synthesis at 9 h. (**B**) Northern blot analysis of cytoplasmic RNA extracted from control (pMo) and E7-expressing (E7/2) cells at 6, 8, 10 and 12 h after release from nocodazole arrest. The 4.0 and 2.8 kb B-*myb* mRNAs and the GAPDH mRNA are indicated.

of complexes is very similar to that seen in mid G_1 extracts made at 8−12 h following release of NIH3T3 cells from serum starvation (Lam and Watson, 1993). It is notable that the pattern of complexes obtained with the 6 h E7/2 extract was markedly different from that found with the control. Hence, while the free E2F complex was readily detectable in the E7/2 extract, there was a virtual absence of higher order complexes containing the p107 protein (Figure 5A).

Similar findings were made when the E2F complexes were compared in whole cell extracts prepared from cycling pMo and E7/2 cells (Figure 5B). It is of interest, however, that while substantially reduced compared with control cells, a bandshift corresponding in mobility to the S phase complex was observed with cycling E7/2 cell extracts (Figure 5B). It has been reported very recently that E7 is associated with the S phase E2F complex in a human cervical carcinoma cell line, Caski cells, which express high levels of the HPV16 E7 protein (Arroyo *et al.*, 1993). In partial support of these findings, we observed here that both E7 and p107 antibodies had a similar effect on the E7/2 S phase E2F complex in mobility shift assays (Figure 5B). Thus, addition of either E7 or p107 antibodies super-shifted essentially all the E7/2 S phase E2F complex while, in contrast, pRb antibodies had no discernible effect in this assay (Figure 5B). Moreover, as expected, E7 antibodies had no effect on bandshifts obtained with the cycling pMo cell extract. These results indicate, therefore, that expression of E7 at high levels in NIH3T3 cells substantially reduced the level of S phase E2F complex as assayed by DNA binding. The reduced level of this complex which remained, however, contained both p107 and E7 protein.

Activation of B-myb transcription correlates with the ability of E7 to interact with p107

The apparent importance of p107 in the regulation of B-*myb* promoter activity prompted us to investigate further the effect of E7 mutants which differ in their ability to interact with pRb and p107 (Figure 6A). Co-transfection experiments in NIH3T3 cells showed that transactivation of the B-*myb* promoter strictly correlated with the ability of E7 mutants to form a complex with p107 (Figure 6B) and that this effect was dependent on the B-*myb* promoter E2F site (Figure 6C).

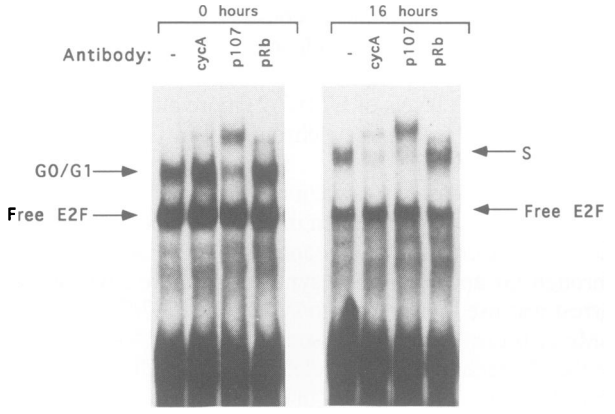

Fig. 4. Identification of the components of E2F complexes in NIH3T3 cells. Whole cell extracts were prepared from untransfected NIH3T3 cells made quiescent by serum starvation (0 h extract: cells were in G_0) and from these cells at 16 h after readdition of serum (16 h extract: cells were in S phase). Alterations in the formation or mobility of the G_0/G_1 and S phase E2F complexes were analysed by electrophoresis on polyacrylamide gels following incubation with antibodies specific for cyclin A, p107 or pRb.

Thus, both the E7 proteins carrying mutations outside the pRb/p107 binding region (PRO2 and GLY58/GLY91) retained the ability to transactivate B-*myb* transcription in this transient assay, consistent with the ability of the PRO2 mutant to elevate the levels of endogenous B-*myb* expression (Figure 2A). A deletion mutant in which the entire pRb/p107 binding domain has been removed (Δ21−35) and a point mutant which fails to bind pRb or p107 *in vitro* (GLY24) were both defective for activation of the B-*myb* promoter, in agreement with the lack of effect of the GLY24 mutant on endogenous B-*myb* expression in NIH3T3 cells (Figure 2A). Interestingly, the point mutant (GLY26), which retains p107 binding activity but shows essentially no pRb binding, functions like wild-type E7 in this assay (Figure 6B). To ensure that these results were not affected by differences in stability of the E7 mutants, parallel cell extracts were analysed by immuno-Western blotting (Figure 6D). Under the transfection conditions used, this demonstrated that the various E7 proteins were expressed

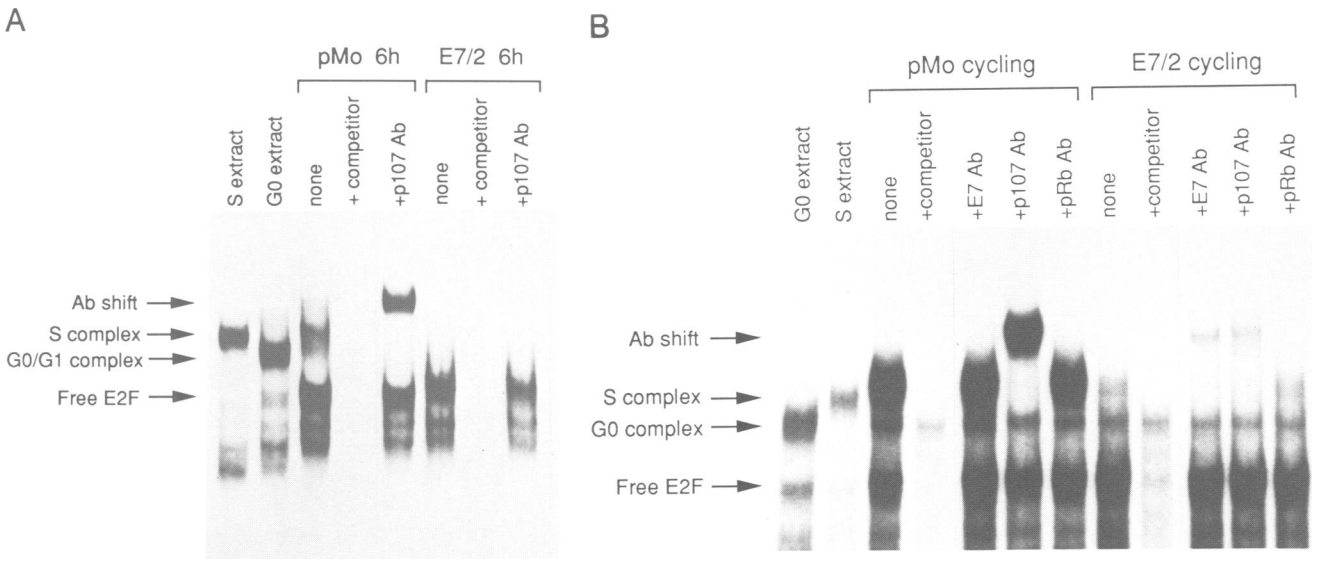

Fig. 5. Analysis of E2F complexes in E7 expressing cells. (**A**) Whole cell extacts were prepared from E7 expressing (E7/2) or control (pMo) NIH3T3 cells at 6 h after release from nocodazole arrest, i.e. when cells were in mid G_1. Proteins binding to a ^{32}P-labelled E2F binding site oligonucleotide (derived from the adenovirus E2 promoter) were detected by the electrophoretic mobility shift assay on polyacrylamide gels. The positions of the previously identified complexes in G_0 and S phase NIH3T3 cell extracts are indicated, as is that of the complex formed with free E2F. Binding reactions with the 6 h pMo and E7/2 cell extracts contained no further addition (none), competitor B-*myb* E2F binding site oligonucleotide (competitor) or the SD15 p107 antibody (p107 Ab). (**B**) Electrophoretic mobility shift assays were similarly performed using whole cell extracts prepared from cycling pMo and E7/2 cells. In this example, bandshifts were obtained using a ^{32}P-labelled mouse B-*myb* oligonucleotide. The competitor oligonucleotide contained the adenovirus E2 promoter E2F binding site. Additions of an HPV16 E7 monoclonal antibody, the SD15 p107 antibody and the XZ104 pRb monoclonal antibody were made to binding reactions.

at reasonably comparable levels. Increased expression of the E7 GLY24 protein compared with the other E7 proteins analysed (Figure 6D) did not affect the interpretation of the results, as this mutant was negative in the transactivation assay (Figure 6B). These results are therefore consistent with the model that transcriptional transactivation by E7 results from the interference with a p107-containing negative regulatory complex at the E2F site of the B-*myb* promoter.

E7 immortalized human keratinocytes show an increased level of B-myb expression

This study had centred on the effect of E7 on mouse B-*myb* expression, since a clear role for E2F mediated regulation of this promoter has only recently been established (Lam and Watson, 1993) and a full characterization of the human B-*myb* promoter remains to be completed. To begin to address the question of whether E7 could deregulate endogenous B-*myb* expression in human cells, the natural target of HPV infections, we examined the levels of B-*myb* mRNA in human foreskin keratinocyte cell lines (101/3 and 104/7) immortalized with HPV16 E6 and E7. These cell lines, which were shown to express E7 protein by immunoprecipitation (data not shown), clearly expressed much higher levels of B-*myb* mRNA than untransfected primary human keratinocytes (Figure 7). Although not definitive, these results provide the first evidence that E7 may deregulate expression of B-*myb* in human as well as mouse cells.

Discussion

This study identifies the B-*myb* promoter as a target for transactivation by HPV16 E7. The cell cycle regulated induction of B-*myb* expression during late G_1 has been shown previously to result from de-repression of transcription inhibition mediated through an E2F binding site in the B-*myb* promoter. A correlation was found here between the ability of E7 mutant proteins to transactivate B-*myb* transcription and their capacity to bind p107. Consistent with this observation, different p107-containing E2F complexes were found to be present in control NIH3T3 cells at the G_0/G_1 and S phases of the cell cycle. Expression of high levels of E7 in NIH3T3 cells had clear effects upon p107/E2F complexes at both stages of the cell cycle.

It is apparent from our results that p107 is a key determinant in cell cycle regulation of B-*myb* transcription in NIH3T3 cells. Previously, it has been shown that p107 may inhibit E2F-dependent transcription in transient transfection assays (Schwarz *et al.*, 1993; Zamanian and La Thangue, 1993; Zhu *et al.*, 1993). It is evident, however, that p107 does not necessarily act in an inhibitory capacity, since the cyclin A-containing p107/E2F complex is consistently detected in S phase cells in which B-*myb* transcription is maximal. Rather, our data suggest that modification of an inhibitory p107/E2F complex present during G_1 is the critical control point in induction of B-*myb* transcription at the G_1/S boundary in NIH3T3 cells. Such modifications may include, or be the result of, acquisition by p107/E2F of cyclin A and presumably $p33^{cdk2}$ (Cao *et al.*, 1992; Devoto *et al.*, 1992; Shirodkar *et al.*, 1992). A number of other proteins are present in the purified S phase E2F complex, however (Arroyo *et al.*, 1993), and their potential role in induction of B-*myb* transcription remains to be determined. The way in which modification of p107/E2F could lead to loss of repressor activity must remain a matter for speculation at present. It is notable that a similar correlation has recently been described between binding of an apparently distinct cyclin A/p107 complex and transcriptional activation of the human thymidine kinase promoter as cells enter S phase (Li *et al.*, 1993).

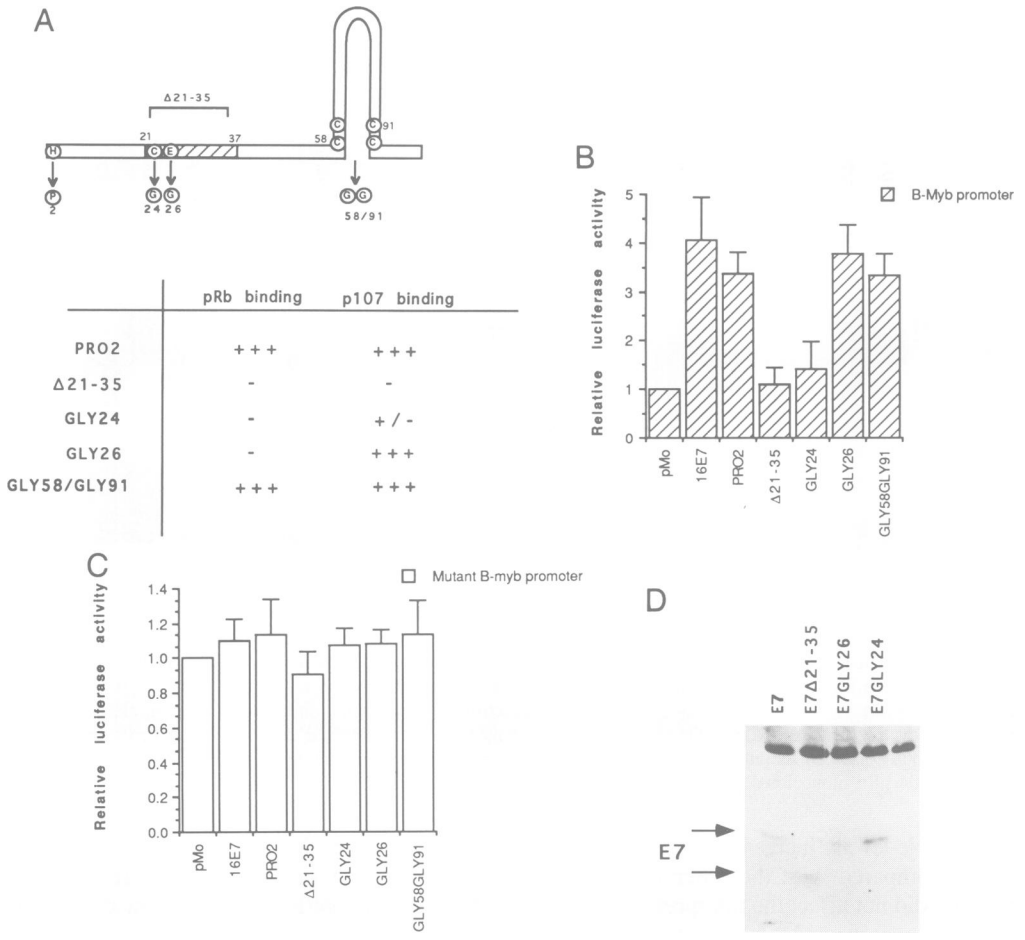

Fig. 6. Correlation of E7 binding activities with transactivation of the B-*myb* promoter. (**A**) Schematic representation of E7 protein showing the conserved cysteine motifs in the C-terminus and the region of similarity with adenovirus E1A and SV40 LTR (residues 21–37) as a hatched box. The effect of point mutations (PRO2, GLY24, GLY26 and GLY58/GLY91) or a deletion (Δ21–35) on *in vitro* associations between E7 and pRb or p107 are summarized below. (**B**) Transactivation of the wild-type B-*myb* promoter following transient co-transfection of the B-*myb* reporter construct, pGL2-(-536), and plasmids expressing wild-type or mutant E7 proteins into cycling NIH3T3 cells. Results are the average of at least four experiments. (**C**) Transactivation of the mutant B-*myb* promoter by wild-type and mutant E7. (**D**) Expression of wild-type and mutant E7 proteins following transient transfection into NIH3T3 cells. Proteins were detected by immunoblot analysis.

No evidence was obtained for the involvement of pRb in regulation of B-*myb* transcription in NIH3T3 cells. It is possible that a pRb/E2F complex does not exist in these cells, although NIH3T3 cells do express pRb detectable by direct immunoprecipitation (R.Davies, unpublished observation). It is of interest that a recent study using Swiss 3T3 cells identified pRb, but not p107, in complexes interacting with the E2F binding site of the murine thymidine kinase promoter (Ogris *et al.*, 1993). These results may therefore indicate a differential activity of pRb and p107 in the regulation of E2F-responsive genes, consistent with a recent report that pRb and p107 inhibit cell growth through related but not identical mechanisms (Zhu *et al.*, 1993). The basis for these differences remains to be determined, although the ability of pRb and p107 to bind distinct E2F proteins may correlate with a specificity in promoters targeted for regulation (Chittenden *et al.*, 1993; Zhu *et al.*, 1993).

Studies carried out *in vitro* indicate that E7 efficiently targets disruption of the pRb/E2F complex but disrupts the p107/E2F complex only at high concentration (Chellappan *et al.*, 1992; Arroyo *et al.*, 1993). Similarly, *in vivo* pRb/E2F complexes appear to be much more sensitive to disruption by E7 than the S phase p107/E2F complex (Chellappan *et al.*, 1992; Pagano *et al.*, 1993). Despite these findings, our results strongly suggest that interaction of E7 with p107 is essential for this oncoprotein to deregulate E2F-dependent transcription of B-*myb*. In this respect, it is notable

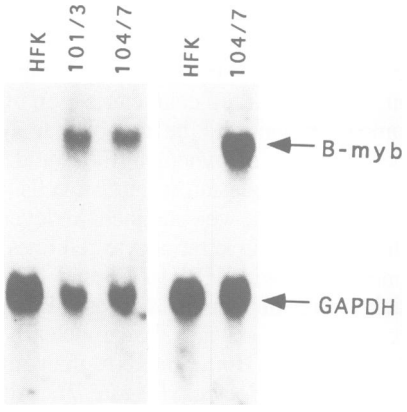

Fig. 7. B-*myb* mRNAs in E7 expressing human cells. RNA was extracted from primary human foreskin keratinocytes (HFK) and immortalized keratinocyte cell lines expressing E6 and E7 (101/3 and 104/7) and was analysed by Northern blotting. The filter was probed simultaneously for B-*myb* and GAPDH expression. The results of probing two different 104/7 RNA preparations are shown so that comparisons with HFK and 101/3 can be more readily made.

that virtually no p107 complexes were detectable in E7/2 cells during G_1 (Figure 5A), the stage of the cell cycle at which B-*myb* transcription is repressed in control cells (Lam and Watson, 1993). Inappropriate transcription of B-*myb* in E7/2 cells during G_1 (Figure 3B), therefore, most likely results from loss of the G_1 p107/E2F complex, which putatively acts as a repressor of transcription. It remains to be determined whether E7 binding to p107 results in disruption of E2F complexes or whether it inhibits their DNA binding properties. Interaction of E7 with the S phase p107/E2F complex would be anticipated to have much less effect upon deregulation of B-*myb* expression, as transcription of this cellular gene is maximal at this stage of the cell cycle. It is notable, however, that the presence of the B-*myb* promoter E2F site allowed E7 to transactivate transcription to levels over and above the constitutively high level obtained with the mutant promoter (Figure 1), suggesting that this effect did not result only from disruption of an inhibitory p107/E2F complex. Moreover, even during S phase it is apparent that expression of endogenous B-*myb* was greater in E7/2 cells than in control cells (Figure 3B). Possibly this additional effect was due to the release of free E2F which could positively transactivate the B-*myb* promoter, although our data do not point to a consistent increase of free E2F in E7 expressing cells (Figure 5). Another possibility, which clearly requires further investigation, is that association of E7 with the S phase p107/E2F complex confers transactivation properties to this complex.

Although the exact function of B-Myb is unknown, it is very likely that it acts as a transcription factor. The strict cell cycle regulation of B-*myb* expression, and its importance for cell growth (Arsura *et al.*, 1992; Sala and Calabretta, 1992), suggest that B-Myb may control entry into S phase, perhaps by targeting transcription of genes required for DNA synthesis (Sala and Calabretta, 1992). Expression of E7 can induce DNA synthesis in serum starved rodent cells (Sato *et al.*, 1989; Banks *et al.*, 1990a), and the ability to interact with pRb and p107 is necessary and may be sufficient for this activity (Banks *et al.*, 1990b). The present study indicates that at least part of this effect could be mediated by deregulation of B-*myb* expression. As HPV DNA replication depends on the host cell replicative machinery, yet these viruses infect epithelial cells which normally stop dividing and follow a programme of terminal differentiation, it is likely that the ability of E7 to induce cellular DNA synthesis plays an important role in virus propagation. This simple hypothesis has been complicated by a recent study indicating that a cotton-tailed rabbit papillomavirus genome containing a mutation in E7 which greatly reduces pRb binding retains the ability to replicate and give rise to warts (Defeo-Jones *et al.*, 1993). Interestingly, this study did not analyse the effects of the E7 mutation on p107 binding or the ability of this mutant E7 protein to induce DNA synthesis.

Although the ability of E7 to complex with p107 and thereby induce expression of E2F-regulated genes might play an important role in viral replication, it is clearly not sufficient for the oncogenic potential of this viral protein; several E7 mutations which do not affect these activities nevertheless abolish transformation. Such mutations include p26GLY (which is pRb binding defective), mutations at the N-terminus of the protein (PRO2) and mutations within the cysteine motifs which coordinate zinc binding (GLY58/GLY91). It is of interest that recent studies have indicated that mutations within the E7 zinc binding region permit interaction with pRb yet prevent the dissociation of pRb/E2F complexes (Huang *et al.*, 1993; Wu *et al.*, 1993), indicating that E2F and E7 bind to slightly different regions of pRb. In contrast, our data showing that the E7 GLY58/GLY91 mutant is able to transactivate B-*myb* expression (Figure 6B) suggest that p107–E2F interactions can be affected by E7 proteins with C-terminal mutations.

The immortalization of human keratinocytes does not appear to require interaction of E7 with either pRb or p107 (Jewers *et al.*, 1992), although whether these functions can contribute to the malignant progression or altered differentiation potential of these cells remains unknown. Our initial studies in human cells (Figure 7) do suggest, however, that E7 also disrupts the normal control of B-*myb* expression in genital keratinoctyes, the natural target cell for HPV infection. In this regard it is of interest that analysis of the human B-*myb* promoter (E.Lam, J.Bennett and R.Watson, manuscript in preparation) revealed evolutionary conservation of the promoter E2F site implicated in cell cycle control and we have evidence that human B-*myb* expression is cell cycle regulated similarly to the mouse gene. Previous studies have indicated that E7 can target complexes containing E2F/pRb and E2F/cyclin A in human cell extracts (Chellappan *et al.*, 1992; Pagano *et al.*, 1992; Arroyo *et al.*, 1993; Wu *et al.*, 1993), and it will be of interest to determine which, if either, is involved in the regulation of human B-*myb* expression.

B-*myb* is the first example of a cell gene regulated by E7, and provides a further step in the pathway by which this viral oncoprotein interferes with the normal regulation of cell cycle progression. This observation is of particular importance since not all potentially E2F responsive genes are regulated in this way by E7. Several studies have failed to show any effect of E7 expression on transcription of c-*myc*, for example (Münger *et al.*, 1991; J.Morris, unpublished observations). Interestingly, adenovirus E1A has been shown to transactivate c-*myc* transcription through the E2F sites (Hiebert *et al.*, 1989), and it will be instructive to determine whether E1A and E7 target similar E2F complexes. We are presently investigating the responsiveness to E7 of other genes which may be important in controlling the G_1/S phase transition.

Materials and methods

Cell culture and synchronization

NIH3T3 cells and their transfected derivatives were cultured in Dulbecco's modified Eagle's medium supplemented with 10% donor calf serum (DMEM/10% CS). Lines were selected following transfection with wild-type and mutant E7 plasmids as described previously (Edmonds and Vousden, 1989). Human foreskin keratinocytes were cultured in keratinocyte growth medium (Clonetics Corp., CA). Primary cells and E6/E7 immortalized cells were established as previously described (Hawley-Nelson *et al.*, 1989) and E6 and E7 expression was confirmed by immunoprecipitation. Transformation, as assayed by anchorage independent growth, was analysed as described previously (Edmonds and Vousden, 1989).

NIH3T3 cells were arrested in G_0 by culture in DMEM/0.5% FCS for 2 days, and were released into the cell cycle by addition of DMEM/10% CS. NIH3T3 cells were synchronized in G_2/M by addition of nocodazole to 0.3 ng/ml (Lam *et al.*, 1992) and the cell cycle status of samples was assessed as described previously (Davies *et al.*, 1993). Cytoplasmic RNA was prepared for Northern blot analysis and probed with mouse B-*myb* and GAPDH probes as described by Lam *et al.* (1992).

Transfection and reporter gene assays

The structure of the mouse B-*myb* promoter luciferase reporter, pGL2-(-536), and that carrying the E2F binding site mutation, pGL2-(-536)mut, have been

described previously (Lam and Watson, 1993), as have the E7 expression plasmids (Davies *et al.*, 1993). Calcium phosphate coprecipitates containing 5 μg of the B-*myb* reporter plasmids, 10 μg of the E7 expression plasmids and 2 μg of a β-galactosidase control plasmid (pJ4Ωβ-gal) were introduced into NIH3T3 cells as described previously (Lam and Watson, 1993); luciferase and β-galactosidase assays were performed on cell extracts made 2 days after transfection (Lam and Watson, 1993).

Detection of E7 by immunoblotting

Immunoprecipitations to detect E7 proteins were carried out with metabolically labelled cell extracts as previously described (Edmonds and Vousden, 1989). Transiently transfected cells were harvested for immunoblot analysis from parallel plates processed for luciferase assays. Immunoprecipitations were carried out as above using a rabbit polyclonal antibody against HPV16 E7 protein (Morris *et al.*, 1993). After resolution on a 15% SDS–polyacrylamide gel, proteins were transferred to nitrocellulose and E7 proteins were detected by chemiluminescence (ECL: Amersham) using a monoclonal anti-HPV16 E7 antibody (Triton Diagnostics, CA).

Electrophoretic mobility shift assays

Whole cell extracts were prepared (Raychaudhuri *et al.*, 1987), and binding reactions and electrophoretic mobility shift assays were performed (Lam and Watson, 1993), as described previously. Probes comprised 200 pg of a ^{32}P-labelled double-stranded deoxyribonucleotide corresponding to nucleotides −224 to −183 of the mouse B-*myb* promoter sequence (Lam and Watson, 1993) (5′-GGCGCCGACGCACTTGGCGGGAGATAGGAA-AGTGGTTCTGTG) or in some instances an oligonucleotide corresponding to the adenovirus E2 promoter E2F binding site (5′-GATCAGTTTTCG-CGCTTAAATTTGAGAAAGGGCGCGAAACTAG). Competitor oligonucleotides were added at a 100- to 1000-fold molar excess to binding reactions. Where appropriate, antibodies were added to binding reactions prior to addition of the DNA probe. These comprised the SD15 p107 monoclonal antibody (obtained from N.Dyson), rabbit polyclonal antibodies to human cyclin A (Pines and Hunter, 1990), the XZ104 pRb monoclonal antibody (Hu *et al.*, 1991) and an HPV16 E7 monoclonal antibody (Triton Diagnostics, CA).

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